Mycoplasma indiense sp. nov., Isolated from the Throats of Nonhuman Primates

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Mycoplasmas isolated from the throats of a rhesus monkey and a baboon within 3 days of their arrival from India were shown to be serologically distinct from 104 previously recognized *Mycoplasma* and *Acholeplasma* spp. Two mycoplasma colonies were cloned and examined in detail for morphology, growth, and biochemical characteristics. The two strains were closely related and had the following properties: guanine-plus-cytosine content of 32 mol%, requirement for sterol, arginine hydrolysis, and anaerobic growth. Glucose was not metabolized, and urea was not hydrolyzed. Strain 3T (= NCTC 11728) is the type strain of a new species, *Mycoplasma indiense*.

Several species in the class *Mollicutes* have been isolated from primates, and some of these species have been isolated from both human and nonhuman primates (3, 6, 17, 19, 21, 31). Most of the surveys on nonhuman primates were carried out with animals that had been housed for some time under laboratory conditions.

A survey was carried out to examine the mycoplasmal throat flora of various wild primates within 3 days of their arrival in the United Kingdom from India (15). The majority of the strains isolated were identified as *Mycoplasma buccale* and *Mycoplasma salivarium*, but a few were not identified. These isolates belonged to two groups; one group contained strains 21E and 26D, and the other group contained strains 3T^T (T = type strain) and 5T. The members of the first group exhibited one-way cross-reactions with *Mycoplasma faucium*, which is inhibited by antisera to strains 21E and 26D (16). Strains 21E and 26D appear to be closely related to each other and to strain 3539 isolated in Holland (24a). The members of the second group did not cross-react with *M. faucium*. Two additional strains, strain SPF recovered from a marmoset in a closed colony and strain HSPP isolated from a chimpanzee (6), are related to each other and also exhibit one-way cross reactions with *M. faucium* strains, but not with strains 21E and 26D (16).

In this paper I describe the characteristics of two isolates, strain 3T^T, which was isolated from a rhesus monkey (*Macaca mulatta*), and strain 5T, which was isolated from a baboon (*Papio anubis*), and I demonstrate that these strains are distinct from all other mycoplasma species. My description of these strains is in accord with the standards published in 1979 (32).

MATERIALS AND METHODS

Mycoplasma strains. Mycoplasmas were isolated in 1974 and 1975 from the throats of a rhesus monkey and a baboon. A culture of each isolate was cloned to produce a pure culture by using a method involving initial filtration of a broth culture through a 220-nm-pore-size membrane filter, culturing of the filtrate on solid medium, transfer of a single resulting colony to another agar plate, and inoculation of the subsequent growth into broth. This whole procedure was repeated four times; thus, the organisms were filter cloned five times (32). One of the clones (the clone isolated from the

rhesus monkey) was designated strain 3T^T, and the other clone (the clone isolated from the baboon) was designated strain 5T.

Mycoplasma species. Mycoplasma type strains were obtained from the National Collection of Type Cultures, Colindale, England, and from H. Atobe, M. F. Barile, J. M. Bradbury, R. J. Fallon, E. A. Freundt, J. T. Heywood, F. T. W. Jordan, D. E. Jaspar, H. Kirchoff, R. H. Leach, G. J. McGarrity, D. L. Rose, G. Smith, D. Taylor-Robinson, and J. G. Tully.

The following *Mycoplasma* spp. type strains were used: *M. agalactiae* PG2, *M. alkalescens* PG51, *M. alvi* Ilsley, *M. anatis* 1340, *M. anseris* 1219, *M. arginini* G230, *M. arthritis* PG6, *M. bovis genitalium* PG11, *M. bovirhinis* PG43, *M. bovis* PG45, *M. bovoculi* M165/69, *M. buccale* CH-20247, *M. californicum* ST-6, *M. canadense* 275C, *M. canis* PG14, *M. capricolum* California kid, *M. caviae* G122, *M. cavipharyngis* 117C, *M. citelli* RG-2C, *M. cloacale* 383, *M. collis* 58B, *M. columbinasale* 694, *M. columbinum* MMP1, *M. columboreale* MMP4, *M. conjunctivae* HRC581, *M. cricetuli* CH, *M. cynos* H831, *M. dispar* 462/2, *M. edwardii* PG24, *M. ellychniae* ELCN-1, *M. equigenitalium* T37, *M. equirhinis* M432/72, *M. fastidiosum* 4822, *M. faucium* DC333, *M. felifaucium* PU, *M. feliminutum* Ben, *M. felis* CO, *M. fermentans* PG18, *M. flocculare* MS42, *M. gallinaceum* DO, *M. gallinarum* PG16, *M. gallisepticum* PG31, *M. gallopavonis* WR1, *M. gateae* CS, *M. genitalium* G-37, *M. glycophilum* 486, *M. hominis* PG21, *M. hyopharyngis* H3-6BF, *M. hyopneumoniae* J, *M. hyorhinis* BT57, *M. hyosynoviae* S16, *M. iners* PG30, *M. iowae* 695, *M. lactuca* 831-C4, *M. leocaptivus* 3L2, *M. leopharyngis* LL2, *M. lipofaciens* R171, *M. lipophilum* MaBy, *M. luminisum* PIMN-1, *M. lucivorax* PIPN-2, *M. maculosum* PG15, *M. melaleuca* M1, *M. meleagridis* 17529, *M. moatsii* MK405, *M. mobile* 163K, *M. molare* H542, *M. muris* RIII4, *M. mustelae* MX9, *M. mycoides* subsp. *capri* PG3, *M. mycoides* subsp. *mycoides* PG1, *M. neurolyticum* Type A, *M. opalescens* MH5408, *M. orale* CH-19299, *M. ovipneumoniae* Y98, *M. oxoniensis* 128, *M. pirum* HRC70-159, *M. pneumoniae* FH, *M. primateum* HRC292, *M. pullorum* CKK, *M. pulmonis* PG34 (= ASH), *M. putrefaciens* KS-1, *M. salivarium* PG20, *M. simbae* LX, *M. somnilux* PYAN-1, *M. spermatophilum* AH159, *M. spumans* PG13, *M. sualvi* Mayfield (clone B), *M. subdolum*

TB, *M. synoviae* WVU1853, *M. testudinis* 01008, and *M. verecundum* 107.

The following *Acholeplasma* spp. type strains were used: *A. axanthum* S743, *A. cavigenitalium* GP3, *A. entomophilum* TAC, *A. equifetale* N93, *A. florum* LI, *A. granularum* BTS39, *A. hippikon* CI, *A. laidlawii* PG8, *A. modicum* PG49, *A. morum* 72-043, *A. multilocale* PN525, *A. oculi* 19L, and *A. parvum* H23M.

Medium and growth conditions. The culture medium used has been described previously (13, 34) and contained thallium acetate and penicillin. The purified agar used in the growth medium was either Ionagar No. 2 (Oxoid Ltd., Basingstoke, England) or Lab M agar (Lab M, London, England). Depending on their biochemical activities, the mycoplasmas were grown in liquid medium containing 1% (wt/vol) glucose (pH 7.8) or in liquid medium containing 0.5 to 1% (wt/vol) arginine (pH 7.3). The species grown in medium containing arginine were not inhibited by 1% arginine. Agar cultures were incubated at 35 to 37°C either in a humid chamber or under anaerobic conditions in a GasPak system. Liquid cultures were stored at -70°C in ampoules.

Growth requirements and characteristics. The clones were subcultured onto solid medium and incubated for 1 week under both aerobic and GasPak anaerobic conditions at 35 to 37°C. The levels of susceptibility to methylene blue were determined by adding 0.002% (wt/vol) methylene blue to the basal solid medium and comparing the growth of inoculated mycoplasmas with the growth of mycoplasmas cultivated on medium without methylene blue. Lipolytic activity was tested by inoculating mycoplasmas onto basal medium enriched with 10% egg yolk emulsion (9). The incubated plates were examined at 3, 7, and 14 days for visual evidence of lipolysis (clearing) or film production (film and spots).

Absence of reversion. The clones were subcultured by five passages in both solid and liquid media that contained no microbial inhibitors in order to determine whether the organisms reverted to bacterial forms. Agar culture colonies of each clone were also treated with Dienes stain in order to differentiate mycoplasma colonies from bacterial L-forms (35).

Morphological studies. Mycoplasma colonies grown on agar were examined microscopically at a magnification of $\times 100$ after 3, 7, and 14 days of incubation. The colonies were transferred to slides and stained with Giemsa stain (10, 18). Liquid cultures were observed by using dark-field microscopy, and organisms were stained with Giemsa stain (18). Organisms grown in broth were harvested by centrifugation, and the resulting cell pellets were fixed in 2% (vol/vol) glutaraldehyde and postfixed in 1% (wt/vol) osmium tetroxide for 1 h. Thin sections of these preparations were stained with uranyl acetate and lead citrate and examined by electron microscopy (38).

Filtration studies. Cultures (after 24 h of incubation) were diluted 1:10 in liquid medium, and the resulting preparations were filtered and refiltered (with a syringe and slight pressure) through a series of membrane filters (Millipore Corp., Bedford, Mass.) with pore diameters of 220, 300, 450, 650, and 800 nm. The number of colony-forming units per milliliter in each filtrate was determined by plating the filtrate onto agar and was compared with the number of colony-forming units per milliliter in the unfiltered culture dilution.

Sterol dependence. Single colonies of strains 3T^T and 5T were seeded onto serum-free solid media supplemented with 0.5% bovine serum albumin, 0.5% glucose, and 10 μ g of palmitic acid per ml. Cholesterol dissolved in Tween 80 was added to give final cholesterol concentrations of 20, 10, 5,

and 1 μ g/ml. Plates containing no cholesterol were included (7, 8, 28, 37). The mycoplasmas were also subcultured onto the basal agar medium without serum. When growth occurred on any of the serum-free media, single colonies were passaged three times on the same medium to ensure that growth was not due to a carryover of serum from the original culture, which would have given misleading results.

The clones were also tested indirectly for sterol dependence by a paper disk inhibition method (11), using either dried disks that originally contained 0.02 ml of a 1.5% (wt/vol) ethanolic solution of digitonin (Sigma Chemical Co., St. Louis, Mo.) or wet disks that contained 0.02 ml of a 20% (wt/vol) aqueous solution of sodium polyanethanol sulfonate (Koch-Light Laboratories, Ltd., Colnbrook, England) on basal growth medium containing Lab M agar. The widths (in millimeters) of zones of growth inhibition were measured.

Biochemical activity. The clones were examined for metabolism of arabinose, arbutin, cellobiose, dulcitol, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose, and xylose; for hydrolysis of esculin, arginine (1 and 0.1%), and urea; for reduction of methylene blue, resazurin, tetrazolium, and tellurite; and for phosphatase activity (1, 2, 20, 30, 36, 39). Positive and negative controls were used for all of the tests except some of the carbohydrate tests. All of the preparations which yielded negative results were checked for viability of the organism.

Erythrocyte techniques. The clones were examined for hemolytic activity, hemadsorption, and hemagglutination with guinea pig, human, and sheep erythrocytes (1, 22).

Polyacrylamide gel electrophoresis. Electrophoresis was carried out as described by Mouches and Bové (24), using a one-dimensional slab. Centrifuged cell suspensions were dissolved in a solution containing 20% glycerol, 5% 2-mercaptoethanol, and 3% sodium dodecyl sulfate in 0.00625 M Tris buffer (pH 6.8). To a 12.5% acrylamide gel 10- or 25- μ l portions of a cell sample were added. Electrophoresis was performed at room temperature for 1 h at a constant current (20 mA). The gels were stained with Coomassie blue (Sigma).

DNA base composition. DNA was extracted from centrifuged broth culture deposits of strain 3T^T by using the method of Gross-Bellard et al. (12), and the guanine-plus-cytosine content was determined from the buoyant density

FIG. 1. Colonies of strain 3T^T grown under anaerobic conditions for 3 days on an agar medium. Bar = 50 μ m.

of the DNA in cesium chloride by ultracentrifugation (29). DNA extracted from *Escherichia coli*, which had a known guanine-plus-cytosine content, was included as a control.

Serological studies. Antisera were prepared as described by Morton and Roberts (23) and Hill (13). The following three serological methods were used: growth inhibition tests with antiserum-impregnated sterile paper disks (5), metabolism inhibition tests in microtiter plates (14, 26, 27, 33), and immunoperoxidase tests with colonies grown on agar (25). All of the tests were carried out in duplicate. Strains 3T^T and 5T were reacted with antisera prepared against the species listed above. Antisera to the two strains were tested with the previously described *Mycoplasma* and *Acholeplasma* species. Antisera to the two strains were also reacted with each other to demonstrate their relationship.

RESULTS AND DISCUSSION

Colonies of strains 3T^T and 5T became visible during primary isolation after 7 to 8 days of incubation under anaerobic conditions, but growth was more rapid after several passages and became visible after 3 days. No growth was visible aerobically even after prolonged incubation or after many passages on the mycoplasma culture medium. The colonies (Fig. 1) had a typical fried egg appearance. No growth was observed on methylene blue agar or on medium without serum. A film was produced on egg yolk agar, and the clones showed lipolytic activity. Neither clone reverted to a bacterial form when it was subcultured on medium without bacterial inhibitors. The colonies rapidly stained with Dienes reagent, suggesting that these isolates were true mycoplasmas (*Mollicutes*) rather than bacterial L-forms (35).

No motility was observed when the strains were examined by using dark-field microscopy, and helical forms were not seen. Liquid cultures stained with Giemsa stain contained pleomorphic forms characteristic of mycoplasmas. The ultrastructure of the organisms was typical of mycoplasma morphology when thin sections were viewed by electron microscopy (4). The cells were bounded by a single trilaminar unit membrane and lacked any evidence of a cell wall (Fig. 2).

Filtration of a broth culture of strain 3T^T reduced the viable count from 6×10^6 CFU/ml in the original dilution to 8×10^5 CFU/ml in the 300-nm-pore-size membrane filtrate and 2×10^4 CFU/ml in the 220-nm-pore-size membrane filtrate.

Strains 3T^T and 5T could not be cultured on medium without cholesterol, but they grew and were passaged on medium containing 5 µg of cholesterol per ml. They were susceptible to digitonin and sodium polyanethol sulfonate

FIG. 2. Electron micrograph of a section of strain 3T^T stained with uranyl acetate and lead citrate. Bar = 100 nm.

(zones of growth inhibition, 8 and 10 mm, respectively); under these conditions growth was semiconfluent.

The biochemical activities of the two strains were identical and are summarized in the species description below. Only arginine was hydrolyzed. In appropriate tests both strains were hemolytic for guinea pig, human, and sheep erythrocytes but did not hemadsorb or hemagglutinate these cells.

The two strains produced similar electrophoretic protein patterns, and these patterns were distinct from the patterns produced by other arginine-hydrolyzing primate mycoplasmas.

The guanine-plus-cytosine content of strain 3T^T was determined from its buoyant density in cesium chloride to be 32 mol%. This value is within the range of values obtained for the previously characterized *Mycoplasma* species.

The serological techniques used revealed similar levels of cross-reactivity between the two strains (Table 1). No significant cross-reactions were detected with any of the species listed in Materials and Methods.

Taxonomic assignment. Strains 3T^T and 5T belong to the family *Mycoplasmataceae* on the basis of their main properties, including absence of cell walls, lack of reversion to bacterial L-forms when the organisms are grown in antibiotic-free media, penicillin resistance, filterability, and production of typical morphology on agar. They belong to the genus

TABLE 1. Serological reactions of *Mycoplasma* spp. and strains 3T^T and 5T with antisera to strain 3T^T and *M. faucium*

Antigen	Results of growth inhibition test (mm) with antiserum to:		Titer in metabolic inhibition test with antiserum to ^a :		Titer in immunoperoxidase test with antiserum to ^b :	
	Strain 3T ^T	<i>M. faucium</i>	Strain 3T ^T	<i>M. faucium</i>	Strain 3T ^T	<i>M. faucium</i>
Strain 3T ^T	6	0	4,096	<32	16,000	<100
Strain 5T	5	0	4,096	<32	8,000	<100
<i>M. faucium</i>	0	3-4	<32	4,096	<100	4,000
All other species ^c	0	NT ^d	<32-64	NT	<100	NT

^a In the metabolism inhibition test the antiserum was diluted 1/16 to 1/32,000.

^b In the immunoperoxidase test the antiserum was diluted 1/100 to 1/16,000.

^c Species listed in Materials and Methods.

^d NT, not tested.

Mycoplasma because they are not strict anaerobes (in contrast to *Anaeroplasma* spp.), are nonhelical (in contrast to *Spiroplasma* spp.), and depend on sterol for growth (in contrast to *Acholeplasma* spp.) and because urease activity could not be demonstrated (in contrast to *Ureaplasma* spp.).

Strains 3T^T and 5T belong to the same species because they have identical biological characteristics and produce identical serological and protein patterns. As there were no significant cross-reactions with any of the previously recognized *Mycoplasma* spp. (see above), strains 3T^T and 5T belong to a new species. The name *Mycoplasma indiensis* sp. nov. is proposed for these organisms.

Description of *Mycoplasma indiensis* sp. nov. *Mycoplasma indiensis* (in.di.en'se. N. L. gen. *indiensis*, pertaining to India, where the infected primates originated).

Morphological and physical characteristics. Pleomorphic cells bounded by a single unit membrane. Colonies on agar have a typical fried egg appearance. Organisms are filterable through 220-nm-pore-size membrane filters and are resistant to penicillin.

Growth characteristics. Requires sterol, but is inhibited by digitonin and sodium polyanethol sulfonate. Optimal growth occurs at 35 to 37°C under anaerobic conditions. Produces a film and exhibits lipolytic activity on agar.

Metabolic characteristics. Glucose is not metabolized; arginine (but not urea) is hydrolyzed.

Serological characteristics. Serologically distinct from all previously described *Mycoplasma* species.

DNA base composition. The DNA base composition is 32 mol% guanine-plus-cytosine.

Habitat. The habitat is nonhuman primate throats.

Type strain. The type strain is strain 3T (= NCTC 11728).

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